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Effects of 8-Methoxypsoralen and Ultraviolet Radiation on Human Lymphoid Cells *in Vitro*

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Oral 8-methoxypsoralen (8-MOP) plus high-intensity long-wavelength ultraviolet radiation (UV-A) is used clinically to induce remissions of psoriasis and mycosis fungoides. Leukocytes in 8-MOP containing blood receive UV-A exposure when circulating through the dermis during therapy. The present study utilizes an *in vitro* assay system to permit quantitation and correlation of multiple biological and physical alterations in human lymphoid cells induced by 8-MOP plus UV-A treatment. Additive inhibition of lymphoid cell DNA synthesis by 8-MOP (0.01 to 1 μg/ml) plus UV-A (1,000 to 29,000 J/m²) was accompanied by a synergistic potentiation of cell killing in the therapeutic exposure range. Reduction in tritiated thymidine (^{3}HTdR) incorporation to 65-70% of control value was associated with normal survival; while ^{3}HTdR incorporation of less than 50% of control induced by any 8-MOP plus UV-A combination tested was associated with less than 10% survival. 8-MOP-DNA-cross-links were detected by the alkaline elution assay only when ^{3}HTdR incorporation was reduced to less than 50% of control. The relative number of crosslinks increased proportionately with further 8-MOP plus UV-A-induced reduction in ^{3}HTdR incorporation. 8-MOP plus UV-A induced at most approximately a 2-fold increase in sister chromatid exchanges (SCE) per chromosome in lymphocytes or lymphoblastoid cells. Increasing 8-MOP plus UV-A exposure resulted in marked toxicity with few cells progressing to second division metaphases and no further increase in SCE's per chromosome. Addition of 13-cis retinoic acid (1 μg/ml) to the lymphoblastoid cells prior to 8-MOP plus UV-A treatment did not significantly alter the ^{3}HTdR incorporation or cell survival. These studies demonstrate that *in vitro* exposure of human lymphoid cells to therapeutic levels of 8-MOP and UV-A may decrease cellular DNA synthesis, produce DNA 8-MOP interstrand cross-links, reduce cell viability and induce small increases in sister chromosome exchanges.

Oral 8-methoxypsoralen (8-MOP) plus high-intensity long-wavelength ultraviolet radiation (UV-A) is being used clinically to induce remissions in psoriasis [1–3] and in mycosis fungoides [4]. 8-MOP when activated by UV-A binds to DNA forming 8-MOP-DNA adducts [5] which in turn inhibit cellular proliferation. Because the 8-MOP is present in the blood following oral administration [6,7] and UV-A penetrates through the epidermis [8], circulating blood cells might be subjected to 8-MOP-DNA photoreactions. Previously, decreased DNA synthesis in circulating leukocytes from some psoriasis patients receiving 8-MOP plus UV-A (PUVA) therapy was reported [9,10].

An *in vitro* assay system was developed to facilitate examination of multiple effects of 8-MOP and UV-A on fresh human lymphocytes and on transformed lymphocyte cell lines [7]. The studies presented here permit quantitation and correlation of multiple 8-MOP plus UV-A induced biological and physical alterations in the same assay system. This investigation demonstrates that inhibition of DNA synthesis by UV-A and 8-MOP is additive, extends the observation of dose dependence of inhibition of DNA synthesis and of cell survival to low therapeutic concentrations of 8-MOP, and describes the relationship of DNA synthesis inhibition to cell killing and to DNA-8-MOP cross-link induction. Additional observations report sister chromatid exchange induction *in vitro* by 8-MOP plus UV-A and the lack of significant effect of 13-cis retinoic acid on 8-MOP plus UV-A induced DNA synthesis inhibition and cell killing.

MATERIALS AND METHODS

Cells

Fresh lymphocytes were obtained from normal donors by leukophoresis (performed by the Red Cross Blood Bank, Washington, D.C.) and further purified by flotation in lymphocyte separation medium (LSM solution, Litton Bionetics, Kensington, MD). An Epstein-Barr virus-transformed lymphoblastoid cell line (E-1) from a normal donor was obtained from Dr. Alan Andrews, Dermatology Branch, National Cancer Institute. The cells were cultured in RPMI 1640 medium (Grand Island Biological Co., Buffalo, NY or B & B Laboratories, Baltimore, MD) supplemented with 17% fetal calf serum (Gibco, or HEM Research, Rockville, MD) without antibiotics, in closed 490 cm² plastic tissue culture roller flasks (Corning No. 25130) at 37°C with continuous rotation. This procedure results in a mild blastogenic response in the fresh lymphocytes [11]. The stock lymphoblastoid cells were fed at 1 to 3 day intervals to maintain exponential growth.

8-MOP Treatment

For irradiation 1,000,000 cells/ml (lymphocytes) or 200,000 cells/ml (E-1) in 75 cm² plastic flasks (Costar Plastics) were suspended in a salt solution consisting of the salts and glucose present in RPMI 1640 medium (NaCl 6.0 g/l, KCl 0.4 g/l, Sodium Bicarbonate 2.0 g/l, $Na_2HPO_4.7H_2O$ 1.5 g/l Calcium Nitrate.$4H_2O$ 0.1 g/l, Magnesium Sulfate. $7H_2O$.0.1 g/l, and Glucose 2.9 g/l). 8-MOP (a gift from Paul B. Elder Co., Bryan, OH) was stored frozen in liquid nitrogen in the dark as a 1 mg/ml stock solution in absolute ethanol. The 8-MOP was diluted in salt solution immediately before addition to the cell suspension and incubated with the cells at 37°C for at least 15 min before UV-A exposure. All manipulations were performed under gold fluorescent lamp (Westinghouse F40 Go, or General Electric F20 T12 Go) illumination.

Irradiation

The flasks containing the cell suspensions were placed horizontally in a holder above a plate glass filter (6-mm thick) which was suspended

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Abbreviations:
^{3}HTdR: tritiated thymidine
8-MOP: 8-methoxypsoralen
PHA: phytohemagglutinin
SCE: sister chromatid exchanges
TPA: tetradecanoylphorbol

above 4 parallel UV-A fluorescent lamps (FR40T12-PUVA-a gift from Phototherapy Lighting Products Group, GTE Sylvania, Danvers, MA). Identical type lamps are used in clinical photo-chemotherapy [1–3] without the plate glass filter.

Figure 1 shows the spectral irradiance of the unfiltered "PUVA" lamp and of the lamp with the plate glass filter. Included is a calculation of the predicted spectral irradiance using pigmented or unpigmented epidermis (data from reference 8) as a filter to mimic the filtering effect of the epidermis on radiation impinging upon lymphocytes in the dermis. Thus, the plate glass filter eliminates radiation below 320 nm which is normally filtered by the epidermis *in vivo.*

The lamp source was described previously [7]. The calculated irradiance within the flask was 22.5 $J/m^2/s$ with an estimated uncertainty of ± 25%. Radiant intensity varied approximately 9% across the exposure field. The cells were kept in suspension during irradiation and exposed to different portions of the field by shaking the plate glass at approximately 40 revolutions per min. Control unirradiated suspensions were placed in an opaque box on the same shaker.

DNA Synthesis

Five replicate 1 ml samples of the cell suspension from each flask were placed in tightly capped 12 × 75 mm plastic tubes (Falcon No. 2058) with 10 μCi/ml methyl-tritiated thymidine (^{3}HTdR) (21–36 Ci/mM ICN Pharmaceuticals, Irvine, CA) and incubated in the dark for 2 hr in a 37°C water bath. ^{3}HTdR incorporation into DNA was measured by a modification [7] of the Millipore assay technique of Robbins et al [12]. Samples were dripped gently onto the Millipore filters, washed successively with saline, 5% trichloroacetic acid, and ethanol; vacuum dried, and counted in RPI Scintillator PPO-POPOP (Research Products International Corp., Elk Grove Village, IL) in Beckman model LS 250 Liquid Scintillation System. In preliminary experiments (Fig 2), a temperature dependence of the rate of ^{3}HTdR incorporation into the lymphoblastoid cells was noted. Greatest activity was measured at 37°, the highest temperature tested. Thus, subsequent experiments were performed with the cell suspensions maintained at 37 ± 3° by use of hot air heaters as monitored with a thermoprobe (Tele-Thermometer model 44TD with probe model 401, Yellow Springs Instrument Co., Yellow Spring, OH).

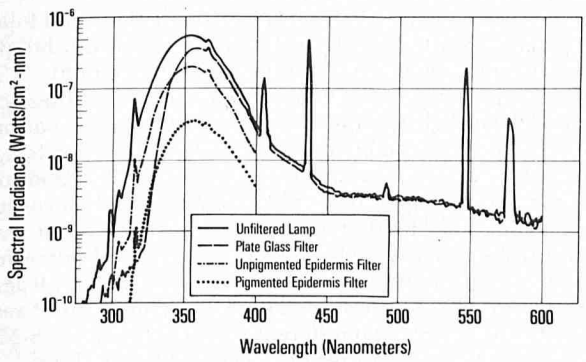

FIG 1. Filtering of "PUVA" fluorescent lamp radiation by plate glass or by epidermis. The spectral irradiance of Sylvania FR40T12 PUVA lamp operated at 115 v was measured at a distance of 2 meters with (—) and without (-) 6-mm plate glass filter. Predicted spectral irradiance using unpigmented epidermis (-·-·-) or pigmented epidermis (. . .) as a filter (data from reference 8) is also shown. Further details in Materials and Methods.

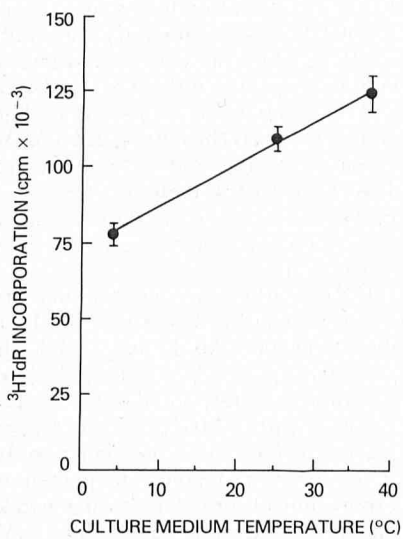

FIG 2. Effects of pretreatment at different temperatures on DNA synthesis in human lymphoblastoid cells. Cells were suspended in culture medium at the indicated temperature for 30 min, ^{3}HTdR was added, and each culture medium was incubated for an additional 2 hr at 37° before harvesting. Further details in Materials and Methods.

Statistical Analysis

For each experiment the mean ^{3}HTdR incorporation determinations were normalized to the untreated control value. Using the method of least squares, the curve $y=a \exp(bx^c)$ was fit to the data for each concentration of 8-MOP where y denotes the normalized ^{3}HTdR incorporation value and x denotes the dose of UV-A. At every exposure level of UV-A a linear regression analysis was performed using y as the dependent variable and x as the independent variable.

Growth Curve Analysis

Growth assays of lymphoblastoid cells: After irradiation a solution containing vitamins, amino acids, phenol red, and fetal bovine serum was added to each flask so that the final medium concentration was 1 × RPMI 1640 with 17% fetal calf serum and 100,000 lymphoblastoid cells/ml. The flasks were placed in a 37°C incubator with 5% CO_2 and 95% relative humidity (Hotpack model 351920), in the dark for 4-14 days without medium change. The concentration of viable cells was estimated daily by duplicate hemocytometer counts of trypan blue (Trypan Blue Stain 0.4% in normal saline, Grand Island Biological Co.) excluding cells. The standard deviation of the duplicate counts was generally less than 10% of the mean. Before treatment greater than 90% of the cells excluded trypan blue.

Survival was estimated by a modification [13] of the method of Alexander and Mikulski [14]. The growth curve of the concentration of viable cells was extrapolated back to zero time along the exponentially increasing portion. This method measures the proportion of the treated cell population which survives with the capacity to proliferate at a normal rate.

Microtiter Well Growth Assay

A modified limiting dilution assay was used (described in detail in reference 13). Briefly, serial dilutions of treated cells were innoculated into 24 wells of a microtiter plate (Linbro) (0.2 ml culture medium per well) and placed in a CO_2 incubator for 2-3 weeks. The wells were examined under an inverted microscope (Leitz, Diavert) for evidence of cell growth. The fraction of negative wells at each dilution served as a basis for determining the surviving fraction by the Poisson formula.

Retinoic Acid Treatment

13-cis-retinoic acid (Ro4-3780) was obtained from Roche Pharmaceuticals through Dr. G. Peck, Dermatology Branch, National Cancer Institute. Stock solution of 1 mg/ml in absolute ethanol was stored frozen in liquid nitrogen. The 13-cis retinoic acid was diluted in salt solution immediately before addition to the cell suspension and incubated with the cells at 37° for 15 min prior to UV-A exposure.

Sister Chromatid Exchange Determination

Human leukocyte cultures were initiated using 12 ml RPMI 1640 medium supplemented with 17% fetal bovine serum containing 500,000 cells ml. Phytohemagglutinin (Gibco M form) was added at a concentration of 0.1 ml per culture coincidentally with bromodeoxyuridine (BrdU) 30 μg/ml.

For the lymphoblastoid cell line BrdU 10 μg/ml was added to complete culture medium containing 100,000 cells/ml.

All cultures were maintained in complete darkness and incubated at 37° C, 72 hr for leukocytes and 48 hr for lymphoblastoid cells. Colcemid was added at concentrations of 0.055 μg/ml and 0.067 μg/ml at 2 and 4 hr before harvest of leukocytes or lymphoblasts, respectively. Cells were fixed in 3:1 methanol glacial acetic acid and slides were prepared by a standard air dry method.

Slides were processed for sister chromatid exchange analysis using a modification of Kornberg and Freelender's Giemsa staining technique [15]. 50 metaphases of each sample were scored.

Alkaline Elution

Alkaline elution was done as previously described [16–18]. Cells were prelabeled with 2-^{14}-C-thymidine ((0.02 μCi/ml, 60 mCi/mM) and were placed on ice immediately following UV-A exposure. Cells to be evaluated for DNA crosslinking were also exposed to x-irradiation (300R at 0-4°). Cells were then layered on a poly vinyl chloride filter, 2 μ porosity (Millipore Corp., Bedford, Mass.) by gravity filteration. Cells were lysed with 5 ml of a solution of sodium lauroylsarcosinate (0.2% w/v) (Sarkosyl, ICN Pharmaceuticals, Plainview, NJ) 2 M NaCl, 0.02 M ethylenedinitrilo tetracetic acid (EDTA), pH 10.

The residual DNA on the filter was eluted with a solution consisting of 0.04 M H_4-EDTA plus tetraporpyl ammonium hydroxide (RSA Corp., Ardsley, NY) 2% in water pH 12.1. The elution rate was 0.04 ± 0.003 ml/min. Ninety minute fractions were collected, and mixed with 3.3 vol of scintillation fluid (Aquasol, New England Nuclear, Boston, MA) plus 0.75% (V/V) acetic acid to reduce chemiluminiesence. Residual radioactivity on the filter was solubilized by 1 N hydrochloric acid and heat (60°, 1 hr) followed by sodium hydroxide (0.4 N, 1 hr) and the same scintillation fluid was then added for counting.

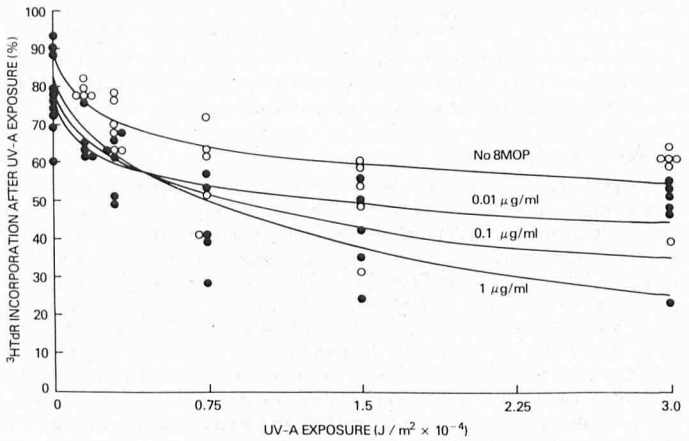

FIG 3. DNA synthesis in human lymphocytes after 8-MOP plus UV-A. Relative effect of UV-A (○) and UV-A plus 0.01 μg/ml 8-MOP (●) on ^{3}HTdR incorporation in lymphocytes from 4 donors. Each *point* represents the mean CPM of the 5 replicate treated cultures divided by the mean CPM of the 5 replicate untreated cultures in the same experiment. The curves were derived by least squares analysis of the points. The curves for 0.1 μg/ml 8-MOP plus UV-A and 1 μg/ml 8-MOP plus UV-A are reproduced from reference 7. The curve for UV-A alone is significantly different from the 0.01 μg/ml 8-MOP plus UV-A curve at every UV-A exposure ($p < 0.05$). Further details as described in the text.

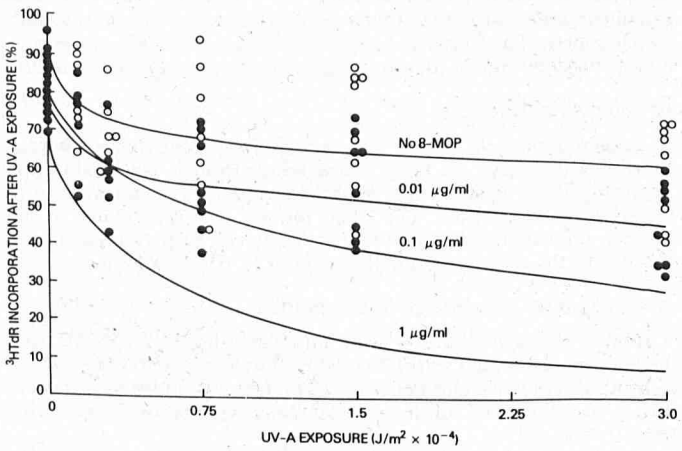

FIG 4. DNA synthesis in human lymphoblastoid cells after 8-MOP plus UV-A. Relative effect of UV-A (○) and UV-A plus 0.01 μg/ml 8-MOP (●) on ^{3}HTdR incorporation in lymphoblastoid cell line E-1. Each *point* represents the mean CPM of 5 replicate treated cultures divided by the mean CPM of the 5 replicate untreated cultures in the same experiment. The curves for 0.1 μg/ml 8-MOP plus UV-A and 1 μg/ml 8-MOP plus UV-A are reproduced from reference 7. The *curve* for UV-A alone is significantly different from the 0.01 μg/ml 8-MOP plus UV-A curve at every UV-A exposure ($p < 0.05$). Further details as described in the text.

Calculation of Crosslink Coefficient

The fraction of DNA retained on the filter is calculated from the amount of the total DNA eluted in the timed fractions. For evaluation of crosslinking, both control and test cells are exposed to 300 R to induce single-strand breaks. When the DNA is subsequently denatured under alkaline conditions, control DNA elutes rapidly. Increased retention of DNA on the filter from cells treated with PUVA plus 300 R indicates DNA crosslinking.

The crosslink coefficient (K_c) is calculated according to the formula:

$$K_c = \left[\frac{1 - R_o}{1 - R_o'}\right]^{1/2} - 1$$

where R_o is the fraction of DNA retained in the 300 R control sample and R_o' is the fraction of DNA retained in the PUVA plus 300 R treated sample both taken at a standard time after elution begins. The derivation of this formula is found in references 17 and 18.

RESULTS

Thymidine Incorporation in Fresh Lymphocytes

The relative effect of UV-A (0-29,000 J/m^2) alone and following 0.01 μg/ml 8-MOP on ^{3}HTdR incorporation into lymphocytes in 11 experiments with four donors is shown in Fig 3. The ^{3}HTdR incorporation in the unirradiated cultures of these cells which had been slightly stimulated by 1 to 4 days incubation in medium containing fetal bovine serum ranged from 796 to 4,000 cpm per 10^6 cells. The 0.01 μg/ml 8-MOP plus UV-A treatment caused a significant ($p < 0.05$) reduction in ^{3}HTdR incorporation in comparison to that caused by UV-A alone at every exposure tested. Figure 3 also contains comparable data from reference 7 for lymphocytes treated with 0.1 μg/ml or 1.0 μg/ml 8-MOP before UV-A exposure. The 0.01 μg/ml 8-MOP result was significantly ($p < 0.05$) different from the 0.1 μg/ml 8-MOP data only for UV-A exposures of 15,000 and 29,000 J/m^2.

Thymidine Incorporation in Lymphoblastoid Cells

Figure 4 shows the relative effect of UV-A (0-29,000 J/m^2) alone and following 0.01 μg/ml 8-MOP on ^{3}HTdR incorporation in 15 experiments with a lymphoblastoid cell line from a normal donor. The ^{3}HTdR incorporation in unirradiated cultures ranged from 41,416 to 263,734 cpm/200,000 cells. The degree of inhibition of ^{3}HTdR incorporation following exposure to UV-A plus 0.01 μg/ml 8-MOP was significantly different ($p < 0.01$) from that after UV-A alone at every exposure tested. Also shown are the results for similar data at 8-MOP concentrations of 0.1 μg/ml and 1 μg/ml from reference 7. There was no significant difference between the 0.01 μg/ml 8-MOP curve and the previous 0.1 μg/ml 8-MOP curve for UV-A exposures of 7,500 J/m^2 or less. As with the lymphocytes (Fig 3), the 0.01 μg/ml 8-MOP curve was significantly different ($p < 0.05$) from the previous 0.1 μg/ml 8-MOP curve only for UV-A exposures of 15,000 and 29,000 J/m^2.

Reductions in DNA synthesis were observed by treatment of lymphoblastoid cells with either UV-A or 8-MOP alone (Fig 4). For most combinations of 8-MOP and UV-A the effect of treatment with 8-MOP plus UV-A on inhibition of ^{3}HTdR incorporation was approximately equal to the sum of the extent of inhibitions observed with 8-MOP and UV-A separately (Fig 5). Evidence suggestive of a weak synergism was observed only with the highest combinations tested. The percent of inhibition of ^{3}HTdR incorporation observed following combined treatment with 0.1 μg/ml 8-MOP plus 15,000 J/m^2 UV-A was approximately equal to the sum of the percent inhibition observed with the separate treatments irrespective of the sequence of the treatments (Table I).

^{3}HTdR incorporation increased to control levels by 48 hr

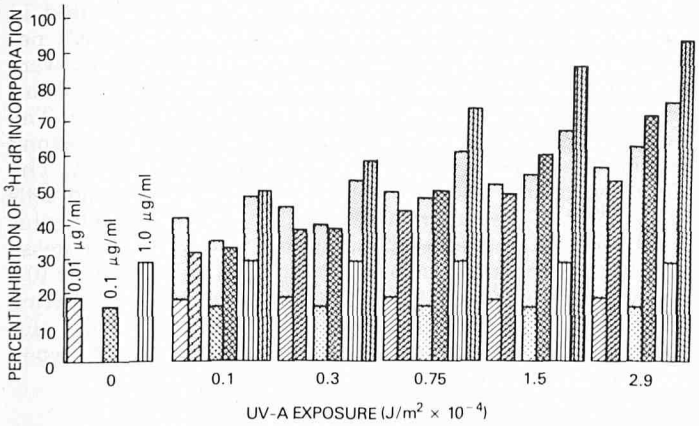

FIG 5. Additivity of DNA synthesis inhibition by 8-MOP plus UV-A in human lymphoblastoid cells. The *curves* from Fig 4 were evaluated at each UV-A exposure for the percent inhibition of ^{3}HTdR incorporation and expressed as *bars*. The *bar* representing the inhibition of ^{3}HTdR incorporation observed by each exposure of UV-A alone was added on top of the *bar* representing inhibition observed by the appropriate concentration of 8-MOP alone and placed next to the bar representing inhibition observed by the combination of 8-MOP plus UV-A. For all except the highest combinations tested, the sum of the heights of the separate bars is not less than the height of the bar representing the combined treatment.

TABLE I. *Effect of sequence of 8-MOP and UV-A treatments on lymphoblastoid cell DNA synthesis*

Treatment		^{3}HTdR Incorporation CPM ± 2SE	Inhibition of ^{3}HTdR Incorporation	Crosslink Coefficient
8-MOP g/ml	UV-A J/m^2			
0	0	123,076 ± 9,334	Control	—
0.1	0	95,763 ± 7,719	22%	0.000
0	15,000	84,666 ± 3,084	31%	0.017
0.1 after UV-A	15,000	53,775 ± 4,805	56%	0.000
0.1 before UV-A	15,000	50,838 ± 7,639	59%	0.086

after treatment in cultures treated with 15,000 J/m^2 UV-A alone, 0.1 μg/ml 8-MOP alone or UV-A followed by 8-MOP. In contrast, ^{3}HTdR incorporation progressively decreased in cultures treated with 0.1 μg/ml 8-MOP followed by 15,000 J/m^2 UV-A so that by 48 hr after treatment ^{3}HTdR incorporation was less than 5% of control cultures (data not shown).

The cell viability was not appreciably altered with respect to the untreated control cells by the 0.1 μg/ml 8-MOP alone, the 15,000 J/m^2 UV-A alone, or the treatment with 8-MOP after UV-A. In contrast, there was a marked inhibition of cell viability induced by the treatment with 8-MOP before UV-A (Fig 6). Measurements of DNA crosslinking by the alkaline elution technique revealed significant crosslinking only in the cells treated with 8-MOP before UV-A (Table I).

DNA-8-MOP Crosslinking

Figure 7 shows the relationship between the ^{3}HTdR incorporation following 8-MOP (1.0, 0.1, or 0.01 μg/ml) plus subsequent UV-A treatment and the relative number of 8-MOP-DNA interstrand crosslinks induced. When ^{3}HTdR incorporation was less than 50% of control there was a dose-dependent increase in 8-MOP-DNA crosslinking detected.

Cell Survival Studies

Figure 8 shows survival of lymphoblastoid cells after treatment with UV-A or with 8-MOP (1.0, 0.1, or 0.01 μg/ml) plus UV-A. The results of 29 experiments are plotted. Survival was measured by extrapolation from growth curves (as in Fig 6) in 7 experiments, by growth in microtiter wells in 2 experiments and by both methods in 10 experiments. There was a UV-A dose-dependent decrease in survival at every 8-MOP concentration. UV-A exposure of 7,500 J/m^2 following pretreatment with 1.0 μg/ml, 0.1 μg/ml or 0.01 μg/ml 8-MOP resulted in survivals of 0.03%, 6%, and 80% respectively. Neither 8-MOP treatment alone nor UV-A exposure alone significantly reduced cell survival.

The relationship between the inhibition of ^{3}HTdR incorpo-

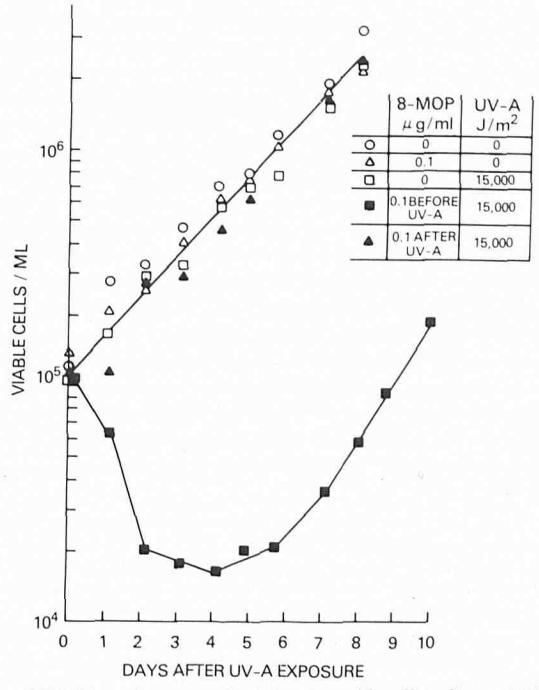

FIG 6. Viability of human lymphoblastoid cells after 8-MOP plus UV-A exposure. Log phase cells were treated with 0.1 μg/ml 8-MOP before (■) or after (▲) exposure to 15,000 J/m^2 UV-A. Control cells were untreated (○), or exposed only to 0.1 μg/ml 8-MOP (△) or to 15,000 J/m^2 UV-A (□). Hemocytometer counts of viable (trypan blue excluding) cells were performed daily following treatment. Further details in Materials and Methods.

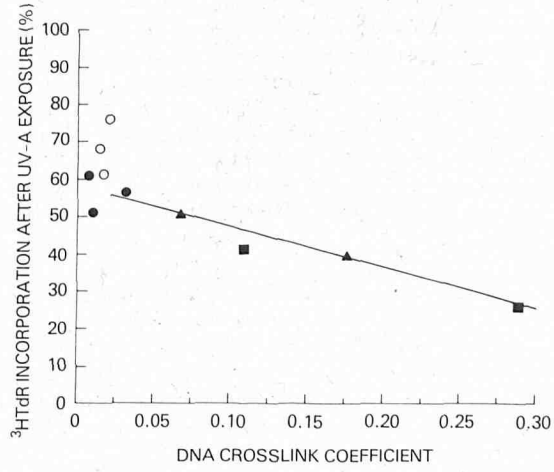

FIG 7. Relationship of DNA synthesis inhibition after 8-MOP plus UV-A to DNA 8-MOP crosslink induction. Lymphoblastoid cells were incubated with 0.01 μg/ml (●), 0.1 μg/ml (▲), 1 μg/ml (■), or no (○) 8-MOP and exposed to UV-A. ^{3}HTdR incorporation was determined from the curves in Fig 4 for each combination of 8-MOP and UV-A. The DNA crosslink coefficient was determined using the alkaline elution assay. Further details are described in Materials and Methods.

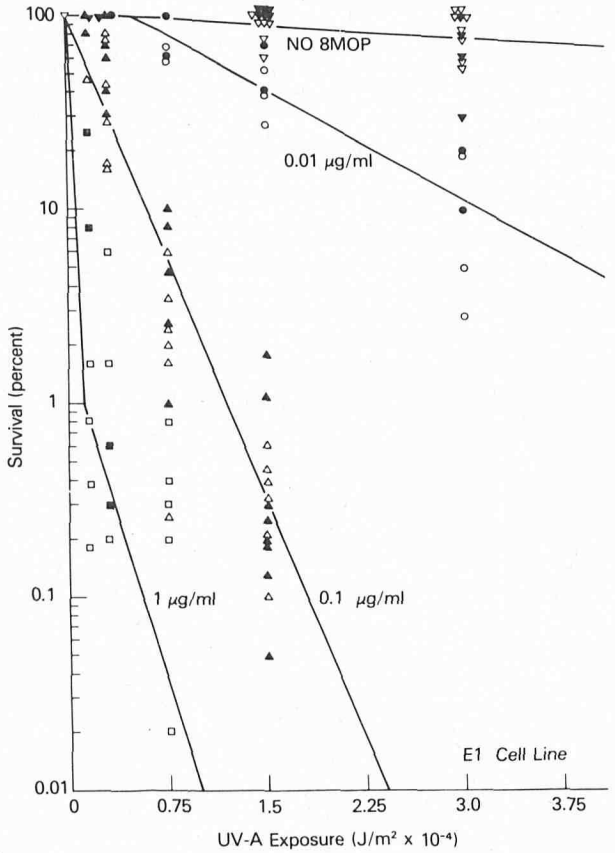

FIG 8. Survival of human lymphoblastoid cells after 8-MOP plus UV-A. Log phase cells were treated with 0.01 μg/ml (○, ●), 0.1 μg/ml (△, ▲), 1 μg/ml (□, ■), or no (▽, ▼) 8-MOP and exposed to UV-A. Survival was measured by extrapolation from growth curves (*closed symbols*) and by growth in microtiter wells (*open symbols*). The curves were calculated by the method of least squares. Further details in Materials and Methods.

ration induced by 8-MOP plus UV-A (from Fig 4) and cell survival (from Fig 8) indicates that survival was unaffected at levels of ^{3}HTdR incorporation of 60 to 70% of control (Fig 9). ^{3}HTdR incorporation less than 60% of control was associated with decreased cell survival. The correlation coefficient between the log of the surviving fraction and the fraction of ^{3}HTdR incorporation remaining was 0.91 over the linear portion of the curve. Similar survival was observed at a similar extent of ^{3}HTdR incorporation inhibition obtained by different combinations of 8-MOP plus UV-A. Thus, 50% of control ^{3}HTdR incorporation was observed with 1 μg/ml 8-MOP plus 3,000 J/m^2 UV-A, with 0.1 μg/ml 8-MOP plus 15,500 J/m^2 UV-A, and with 0.01 μg/ml 8-MOP plus 58,000 J/m^2 UV-A and was associated with approximately 0.5% survival with each combination.

Effects of Retinoic Acid

Preincubation of lymphoblastoid cells with 1 μg/ml 13 cis-retinoic acid was associated with a decrease in ^{3}HTdR incorporation under each of the 4 conditions shown in Fig 10*A*, however, this decrease was not significant at the 0.05 significance level ($p = 0.08$). Survival was unaffected by preincubation with 13 cis retinoic acid prior to 8-MOP, UV-A, or 8-MOP plus UV-A treatment (Fig 10*B*).

Induction of Sister Chromatid Exchanges

The induction of sister chromatid exchanges in lymphocytes after treatment with 8-MOP plus UV-A was significantly increased (Table II). Second division metaphases were analyzed for the frequency of sister chromatid exchanges. Treatment with 0.01 μg/ml 8-MOP plus 7,500 J/m^2 UV-A resulted in approximately a 65% increase in the number of SCE's per metaphase and per chromosome. The lymphocyte cultures were also analyzed for the frequency of first, second, and third division metaphases on the basis of the Giemsa pattern. The frequency of first division metaphases increased and second division metaphases decreased after 8-MOP plus UV-A. The combined regime also increased SCE in lymphoblastoid cells (Table III). However, treatment with 0.1 μg/ml 8-MOP plus 3,000 J/m^2 UV-A resulted in an approximately 2-fold increase in SCE's per chromosome or per metaphase. 8-MOP alone (0.1 μg/ml) or UV-A alone (15,000 J/m^2) were ineffective in altering the number of SCE's. Treatment with 0.1 μg/ml 8-MOP plus 15,000 J/m^2 UV-A substantially reduced the number of second division metaphases.

DISCUSSION

The *in vitro* assay system described approximates some of the conditions of 8-MOP plus UV-A on human lymphoid cells *in vivo* during photochemotherapy. It is designed to measure direct effects of 8-MOP plus UV-A. The UV-A radiation delivered to the cells in *in vitro* was filtered to remove the short-wave ultraviolet radiation which is absorbed by the epidermis before reaching the lymphoid cells circulating through the dermis *in vivo*. The plate glass used in these experiments resulted in a spectral distribution similar to that passing through pigmented epidermis at wavelengths below 330 nm (Fig 1). This short-wavelength ultraviolet radiation (in the UV-B range) is more toxic to leukocytes than the UV-A [19] and

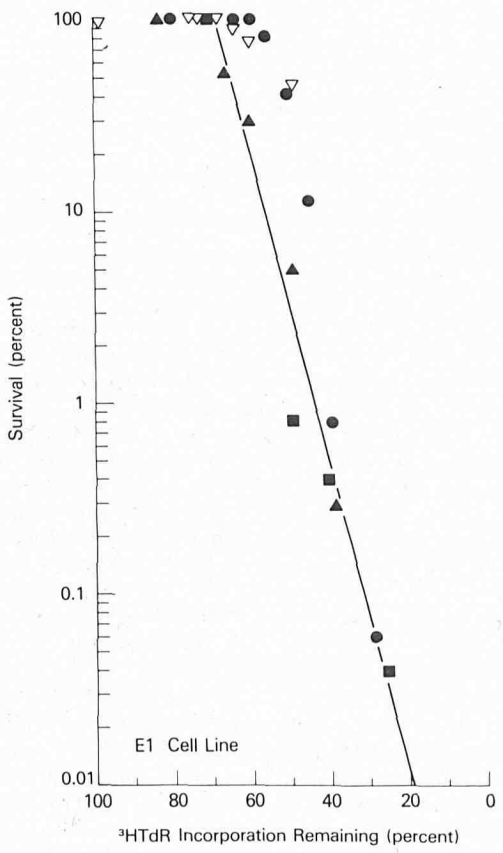

FIG 9. Relationship of human lymphoblastoid cell survival to DNA synthesis inhibition after 8-MOP plus UV-A. The mean survival after treatment with 0.01 μg/ml (●), 0.1 μg/ml (▲), 1 μg/ml (■), or no (▽) 8-MOP plus UV-A was calculated from Fig 8 and compared to the mean determinations of ^{3}HTdR incorporation remaining (in Fig 4). The coefficient of correlation between the log of the surviving fraction and the percent ^{3}HTdR incorporation was 0.91.

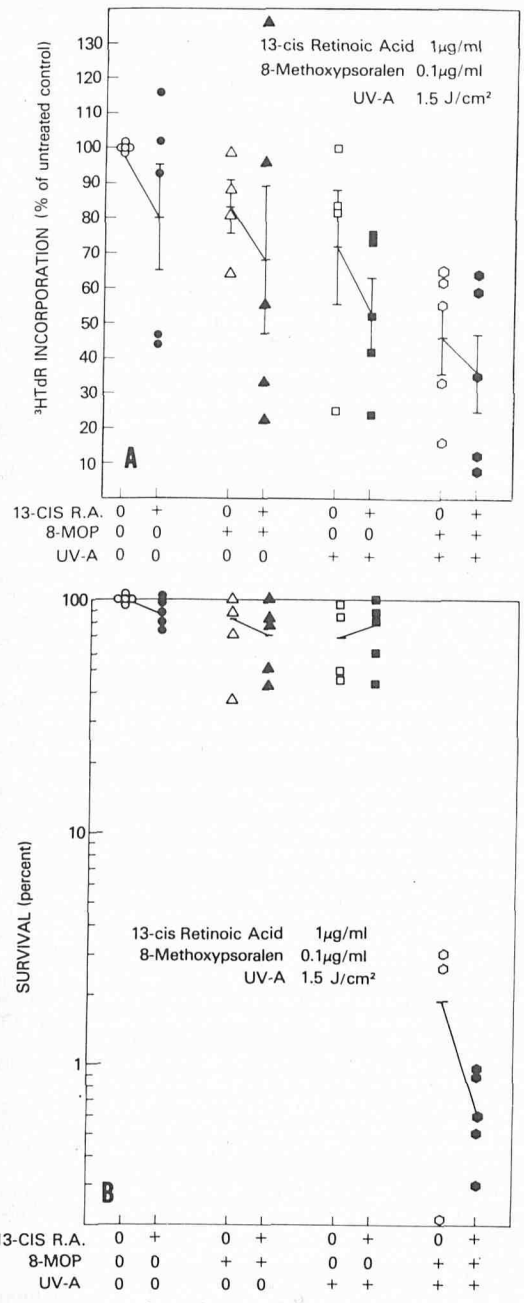

FIG 10. Effect of 13-cis retinoic acid on DNA synthesis and cell survival in human lymphoblastoid cells treated with 8-MOP plus UV-A. Cells were treated with 0.1 μg/ml 8-MOP (△, ▲), 15,000 J/m² UV-A (□, ■), 8-MOP plus UV-A (◇, ◆), or untreated (○, ●) with (*closed symbols*) or without (*open symbols*), exposure to 1 μg/ml 13-cis retinoic acid. *A*, ³HTdR incorporation determined for 2 hr post-treatment (mean ± SE). The difference in ³HTdR incorporation between cultures receiving 13-cis retinoic acid and those not receiving 13-cis retinoic acid was not statistically significant. *B*, Survival measured by microtiter well assay. There was no significant difference in survival between cultures receiving 13-cis retinoic acid and those not receiving 13-cis retinoic acid.

thus its removal is important in seeking to mimic the *in vivo* situation. The *in vitro* assay was performed at 37° for increased DNA synthesis (Fig 2) and to mimic the temperature of circulating lymphoid cells. 8-MOP concentrations used were in the range measured in the serum of patients undergoing photochemotherapy (cited in reference 7). However, no attempt was made to study the effect of circulating metabolites of 8-MOP which are also present in patients [6], or other possible indirect effects of 8-MOP plus UV-A *in vivo*. UV-A exposure to circulating lymphocytes has been estimated to be 1-5% of the skin surface dose [19]. This estimate was based on assuming a cutaneous blood volume of 100–500 ml by analogy to experiments in small mammals. With typical skin UV-A exposure of 100,000 J/m² (10 J/cm²) the circulating lymphocytes would be estimated to be receiving UV-A exposures on the order of 1,000 to 5,000 J/m².

A rapid, dose-dependent inhibition of DNA synthesis occurred with 8-MOP plus UV-A treatment of human lymphocytes (Fig 3) and a lymphoblastoid cell line (Fig 4). Scherer, Kern, and Braun-Falco [20] and Kruger, Christophers, and Schlark [21] measured DNA synthesis 3 days after 8-MOP plus UV-A treatment of phytohemagglutinin (PHA) stimulated leukocytes. They found a qualitativiely similar dose-dependent inhibition of DNA synthesis for the combinnd treatment but no effect with 8-MOP or UVA alone. However, they did not measure immediate inhibition of DNA synthesis. In our studies decreased DNA synthesis was observed when measured immediately after treatment with 8-MOP or UV-A alone, but by 48 hr, DNA synthesis had increased to the control level.

The separate effects of 8-MOP and UV-A treatments on immediate inhibition of DNA synthesis were approximately additive (Fig 5) (for most combinations of 8-MOP and UV-A) and independent of the sequence of treatments (Table I). 8-MOP in the dark is known to bind noncovalently with DNA [5]. UV-A alone has been shown to alter transfer RNA metabolism in bacteria [22]. However, the inhibition of DNA synthesis induced by the lesion(s) resulting from treatment with 8-MOP alone or UV-A alone was nonlethal (Fig 8). Only in those cultures treated with UV-A after 8-MOP was cell survival significantly altered (Fig 6). Further, only cells from those cultures contained detectable DNA interstrand crosslinks.

Survival of lymphoblastoid cells after 8-MOP plus UV-A treatmet was markedly dose-dependent (Fig 8). Depending on the 8-MOP preincubation concentration, survival after a UV-A exposure of 3,500 J/m², presumably in the therapeutic range, varied from 0.3% to 100%. Wulf and Wettermark [23] studying PHA stimulated leukocytes reported similar alterations of cell turnover depending on the 8-MOP concentrations.

We found that long-term cell survival was predictable from the alteration of DNA synthesis measured in the first 2 hr after treatment of the cells with 8-MOP followed by UV-A, irrespective of the 8-MOP concentration (Fig 9). Thus DNA synthesis of 65–70% of the control value or greater was associated with virtually normal survival, while DNA synthesis of less than 50% of the control value was associated with less than 10% survival. There was a similar threshold value for DNA crosslink detection occurring with DNA synthesis less than 50% of the control value. This suggests that DNA crosslinks are associated with lethality. Our previous study [9] detected a greater than 50% reduction in DNA synthesis in circulating leukocytes of pso-

TABLE II. *Sister chromatid exchanges (SCE) induced by 8-MOP plus UV-A in human blood lymphocytes*

Treatment		SCE's per metaphase		SCE's per chromosome	Metaphase frequency		
8-MOP (μg/ml)	UV-A (J/m²)	Range	Mean ± SE		First division	Second division	Third division
0	0	7–14	10.4 ± 0.72	0.23	20%	60%	20%
0	7,500	4–19	12.9 ± 0.86	0.28	30%	56%	14%
0.01	0	7–19	13.2 ± 0.96	0.29	36%	47%	17%
0.01	7,500	9–25	17.3 ± 0.95	0.38	52%	34%	14%

TABLE III. *Sister chromatid exchanges (SCE) induced by 8-MOP plus UV-A in human lymphoblastoid cells*

Treatment		Average No. of chromosomes per metaphase	SCE's per metaphase		SCE's per chromosome
8-MOP (μg/ml)	UV-A (J/m^2)		Range	Mean ± SE	
0	0	91.0	10–31	21.2 ± 1.61	0.23
0	15,000	85.0	10–29	21.0 ± 1.12	0.25
0.1	0	90.5	6–29	19.3 ± 1.36	0.21
0.1	3,000	89.5	17–68	43.9 ± 3.40	0.49
0.1	15,000	No Metaphases Detected			

riasis patients undergoing photochemotherapy when ^{3}HTdR incorporation was measured in blood samples taken immediately after UV-A. A similar reduction in DNA synthesis in leukocytes of photochemotherapy treated patients was reported by Friedmann and Rogers [10]. If the *in vitro* data can be extrapolated to the *in vivo* situation, this would imply that a portion of the circulating leukocytes would be killed following 8-MOP plus UV-A. In this regard Ortonne et al [24], and Haftek et al [25] reported decreased circulating E rosette forming cells and Dahl, Nyfors, and Brodthagen [26] observed a decrease in the percentage of peripheral blood neutrophils in psoriasis patients treated with 8-MOP plus UV-A.

Retinoic acid derivatives have been reported to shorten the clinical course of photochemotherapy in psoriasis patients [27]. Epstein [28] found that in hairless mice, retinoic acid inhibited ultraviolet-induced epidermal hyperplasia as measured by autoradiographic assessment of ^{3}HTdR incorporation. Kensler and Mueller [29] reported that retinoic acid, but not 13 cis retinoic acid, was a potent inhibitor of the increased DNA synthesis induced by tetradecanoylphorbol acetate (TPA) plus PHA in bovine lymphocytes. Thus the mechanism of action of the retinoids in psoriasis patients receiving photochemotherapy could involve a potentiation of the effects of 8-MOP plus UV-A on DNA synthesis or cell survival. In our studies, however, with a short exposure to a single concentration of 13 cis-retinoic acid, in the presumed therapeutic range [30], no significant alteration in DNA synthesis (Fig 10*A*) or cell survival (Fig 10*B*) was observed in comparison to the values observed after treatment with 8-MOP, UV-A or both in the absence of the retinoic acid derivative.

8-MOP plus UV-A treatment of human lymphoid cells *in vitro* has been shown to induce an increased rate of sister chromatid exchanges [31–36]. In the present study at most a 2-fold increase in SCE's after 8-MOP plus UV-A (Tables II and III), was found. In lymphocytes and lymphoblastoid cells, 8-MOP pretreatment followed by UV-A exposure was associated with cell toxicity or mitotic delay as indicated by the smaller fraction of cells attaining second mitoses during the incubation period. With 0.1 μg/ml 8-MOP pre-incubation, 15,000 J/m^2 UV-A followed by BrdU had an adverse effect on cell progression as indicated by the low number of second division metaphases seen in the lymphoblastoid cells. Thus, based on 50 metaphases, there was a very limited range of 8-MOP plus UV-A concentrations in which increases in SCE could be detected; and the increase in SCE frequency was relatively small. In contrast, treatment of lymphocytes with other DNA crosslinking agents may induce an increase in SCE to 5 times control value (mitomycin-C) [36], or to 10 times the control value (busulfan) [37]. Thus in human lymphoid cells, SCE induction by 8-MOP plus UV-A is a relatively insensitive indicator of cell damage. Lambert et al [33] demonstrated a 31% increase in SCE/cell when cells were removed from patients 2 hr after oral administration of 8-MOP subsequently exposed to 18,000 J/m^2 UV-A. Wolf-Schreiner et al [35] found a 34% increase in SCE/cell with a similar protocol using 10,500 J/m^2 UV-A. Since circulating lymphocytes may have received less than 10,000 J/m^2 UV-A, as discussed by Burger and Simons [38] the number of SCE induced per cell may be too low to be detected. Other studies, however, have documented alterations in circulating lymphoid cells of patients treated with 8-MOP plus UV-A. In this regard, Kraemer and Weinstein [9] and Friedmann and Rogers [10] detected decreased DNA synthesis of circulating leukocytes of some psoriasis patients undergoing photochemotherapy and Strauss et al [39] detected an increased frequency of thioguanine resistant circulating lymphocytes during 8-MOP photochemotherapy for vitiligo.

REFERENCES

1. Parrish JA, Fitzpatrick TB, Tanenbaum L, Pathak MA: Photochemotherapy of psoriasis with oral methoxsalen and longwave ultraviolet light. New Engl J Med 291:1207–1211, 1974
2. Wolff K, Fitzpatrick TB, Parrish JA, Gschnait F, Honigsmann H, Pathak MA, Tanenbaum L: Photochemotherapy for psoriasis with orally administered methoxysalen. Arch Dermatol 112:943–950, 1976
3. Melski JW, Tanenbaum L, Parrish JA, Fitzpatrick TB, Bleich HL: Oral methoxsalen photochemotherapy for the treatment of psoriasis: Cooperative clinical trial. J Invest Dermatol 68:328–335, 1977
4. Roenigk HH: Photochemotherapy for mycosis fungoides. Arch Dermmatol 113:1047–1051, 1977
5. Tjerneld F, Norden B, Ljungeren B: Interaction between DNA and 8-methoxy psoralen studied by linear dichroism. Photochem Photobiol 29:1115–1118, 1979
6. Busch U, Schmid J, Koss FW, Zipp H, Zimmer A: Pharmacokinetics and metabolite pattern of 8-methoxypsoralen in man following oral administration as compared to the pharacokinetics in rat and dog. Arch Dermatol Res 262:255–265, 1978
7. Kraemer KH, Waters HL, Ellingson OL, Tarone RE: Psoralen plus ultraviolet radiation-induced inhibition of DNA synthesis and viability in human lymphoid cells in vitro. Photochem Photobiol 30:263–270, 1979
8. Everett MA, Yeargers E, Sayre R, Olson RL: Penetration of epidermis by ultraviolet rays. Photochem Photobiol 5:553–542, 1966
9. Kraemer KH, Weinstein GD: Decreased thymidine incorporation in circulating leukocytes after treatment of psoriasis with psoralen and long-wave ultraviolet light. J Invest Dermatol 69:211–214, 1977
10. Friedmann PS, Rogers S: Photochemotherapy of psoriasis: DNA damage in blood lymphocytes. J Invest Dermatol 74:440–43, 1980
11. Zielski JV, Golub SH: Fetal calf serum-induced blastogenic and cytotoxic responses of human lymphocytes. Cancer Res 37:3842–3846, 1976
12. Robbins JH, Gart JJ, Levis WR, Burk PG: The millipore filter assay technique for measuring tritiated thymidine incorporation into DNA in leucocyte cultures. Clin Exp Immunol 11:629–640, 1972
13. Kraemer KH, Waters HL, Buchanan JK: Survival of human lymphoblastoid cells after DNA damage measured by growth in microtiter wells. Mutat Res, 72:285–294, 1980
14. Alexander P, Mikulski ZB: Differennes in the response of leukaemia cells in tissue culture to nitrogen mustard and to dimethyl myleran. Biochem Pharmacol 5:275–282, 1961
15. Kornberg JR, Freelender EF: Giemsa technique for the detection of sister chromatid exchanges. Chromosoma (Berl) 48:355–360, 1974
16. Kohn KW, Erickson LC, Ewig RAG, Friedman CA: Fractionation of DNA from mammalian cells by alkaline elution. Biochemistry 15:4629–4637, 1976
17. Ewig RAG, Kohn KW: DNA protein cross-linking and DNA interstrand cross-linking. Cancer Res 38:3197–3203, 1978
18. Kohn KW, Ewig RAG, Erickson LC, Zwelling LA: Measurements of strand breaks and cross-links in DNA by alkaline elution, *Handbook of DNA Repair Techniques*. Edited by E Friedberg, P Hanawalt. Marcel Dekker, in press
19. Morison WL, Parrish JA, Anderson RR, Bloch KJ: Sensitivity of mononuclear cells to UV radiation. Photochem Photobiol 29: 1045–1047, 1979
20. Scherer R, Kern B, Braun-Falco O: UV-A-induced inhibition of proliferation of PHA-stimulated lymphocytes from humans

treated with 8-methoxypsoralen. Br J Dermatol 97:519–528, 1977
21. Kruger JP, Christophers E, Schlark LA: Dose-effects of 8-methoxypsoralen and UVA in cultured human lymphocytes. Br J Dermatol 98:141–144, 1978
22. Jagger J: Effects of near-ultraviolet radiation on microrganisms. Photochem Photobiol 23:451–454, 1976
23. Wulf HC, Wettermark G: Toxic effects of 8-methoxypsoralen on lymphocyte devision. Arch Dermatol Res 260:87–92, 1977
24. Ortonne JP, Claudy Al, Alario A, Thivolet J: Decreased circulating E rosette forming cells in psoralen UVA treated patients. Arch Dermatol Res 258:305–306, 1977
25. Haftek M, Glinski W, Jablonska S, Obalek S: T Lymphocyte E rosette function during photochemotherapy (PUVA) of psoriasis. J Invest Dermatol 72:214–218, 1979
26. Dahl KB, Nyfors A, Brodthagen H: Decrease in neutrophils observed in vivo in psoriatics after PUVA therapy. Arch Dermatol Res 262:131–134, 1978
27. Fritsch PO, Honigsmann H, Jaschke E, Wolff K: Augmentation of oral methoxsalen photochemotherapy with an oral retinoic acid derivative. J Invest Dermatol 70:178–182, 1978
28. Epstein J, cited in Kripke ML, Glasman HN: Retinoic acid and photocarcinogenesis workshop. J Am Acad Dermatol 2:439–442, 1980
29. Kensler TW, Mueller GC: Retinoic acid inhibition of the comitogenic action of merezin and phorbol esters in bovine lymphocytes. Cancer Res 38:771–775, 1978
30. Frolik CA, Tavela TE, Peck GL, Sporn MB: High pressure liquid chromatographic determination of 13-cis-retinoic acid and all-trans-retinoic acid in human plasma. Analyt Biochem 86:743–750, 1978
31. Popescu NC, Amsbaugh SC, DiPaolo JA: The relevance of caffeine post-treatment to SCE incidence induced in Chinnse hamster cells. Mutat Res 60:313–320, 1979
32. Carter DM, Wolff K, Schnedl W: 8-methoxypsoralen and UV-A promote sister chromatid exchanges. J Invest Dermatol 67:548–551, 1976
33. Lambert B, Morad M, Bredberg A, Sanbeck G, Thyresson-Hok M: Sister chromatid exchanges in lymphocytes from psoriasis patients treated with 8-methoxypsoralen and longwave ultraviolet light. Acta Dermatovener (Stockh) 58:13–16, 1978
34. Waksvik H, Brogger A, Stene J: Psoralen/UVA treatment and chromosomes. Hum Genet 38:195–207, 1977
35. Wolf-Schreiner EC, Carter DM, Schwarzacher HG, Wolf K: Sister chromatid exchanges in photochemotherapy. J Invest Dermatol 69:387–391, 1977
36. Faed MJW, Mourelatos D: Enhancement by caffeine of sister-chromatid exchange frequency in lymphocytes from normal subjects after treatment by mutagens. Muta Res 49:437–440, 1978
37. Honeycomb JR: The effects of busulphan on the chromosomes of normal human lymphocytes. Mutat Res 57:35–49, 1978
38. Burger PM, Simons J: Mutagenicity of 8-methoxypsoralen and long-wave ultraviolet irradiation in diploid human skin fibroblasts: An improved risk estimate in photochemotherapy. Mutat Res 63:371–389, 1979
39. Strauss GH, Albertini RJ, Krusinski PA, Baughman RD: 6-thioguanine resistant peripheral blood lymphocytes in humans following psoralen, long-wave ultraviolet light (PUVA) therapy. J Invest Dermatol 73:211–216, 1979